

Use of Partially Purified Banana Peel Polyphenol Oxidase in the Degradation of Various Phenolic Compounds and Textile Dye Blue 2RNL

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Abstract

Polyphenol oxidase (PPO) enzyme was isolated from banana peel. Enzyme was partially purified by ammonium sulphate precipitation (70%). The partially purified PPO showed optimum temperature 35 °C and pH 7.0. This PPO enzyme preparation was employed for the degradation of four different phenolic compounds and textile dye Blue 2RNL. PPO degraded 1 g/L gallic acid (100%) in 24 h. PPO also decolorized 40 mg/L Blue 2RNL dye (89%) in 24 h. The decolorization of Blue 2RNL dye by PPO enzyme was evaluated and confirmed by UV-Vis spectroscopy analysis. The treatment of phenolic compounds and textile dye Blue 2RNL with PPO resulted in the remarkable loss of chemical oxygen demand (COD). Also the degradation products showed no toxicity for germination of various plant seeds.

Keywords

Polyphenol Oxidase; Banana Peel; Phenolic Compounds; Degradation; Phytotoxicity

Introduction

Aromatic compounds, such as polyphenols comprise the second largest group of natural products, in

addition to a variety of xenobiotics that are manmade aromatic pollutants [1]. Phenol and phenolic compounds have been listed as priority-pollutants. These are introduced into the environment from a variety of industrial sources [2]. They are frequently used as industrial reagents in the production of rubber, dyes, pesticides, colors, plastics, pharmaceuticals, and cosmetics. Removal of phenols from industrial water effluents is an important practical problem, since many of these compounds are toxic and their presence in drinking and irrigation water is a health hazard [3]. Tremendous efforts have been made worldwide to minimize the pollution of water bodies caused by phenolic effluents. The treatment methods used for removal of phenolic compounds are anodic oxidation, adsorption, photocatalysis, and oxidative catalysis [4]. Among these methods, some have disadvantages like time-consuming, expensive procedures and formation of toxic residues [5].

Another potential source of environmental pollution is textile dyeing industries. Considering both volumes

discharged and effluent composition, the wastewater generated by the textile industry is rated as the most polluting among all industrial sectors [6]. Effluents from textile and dyeing industries have high BOD, COD, color, pH, and also it contains metal ions, hence it is very difficult to treat such effluents [7]. The current states of the art for the treatment of wastewaters containing dyes are physico-chemical techniques, such as flocculation, coagulation, adsorption, membrane filtration, precipitation, irradiation, ozonization and Fenton's oxidation. All of these have serious restrictions as they themselves produce large amount of sludge which may be toxic. Also these processes are not economically feasible [8].

There is a growing recognition that enzymes can be used to target specific pollutants for treatment. An enzymatic treatment is gaining more prevalence over physico-chemical processes for removal of pollutants [9]. In recent years a great deal of research has been directed towards developing processes in which enzymes are employed to remove/transform phenolic compounds and dyes from polluted wastewater. Oxidoreductive enzymes such as peroxidases, laccases and polyphenol oxidases are participating in the degradation/removal of aromatic pollutants from various contaminated sites. These enzymes can act on a broad range of substrates and they can also catalyze the degradation or removal of organic pollutants present in very low concentration at the contaminated sites [10]. Plant peroxidases are catalyzing decolorization of wide spectrum of dyes but they require expensive H_2O_2 as a co-substrate [11]. The rare availability and high cost of purification of laccases limit its use in decolorization studies. Plant polyphenol oxidases are the simple and cheaper alternative for the removal of aromatic pollutants due to its utilization of free molecular oxygen as an oxidant [10]. In the present study an attempt has been made to use partially purified banana peel PPO for the degradation of various phenolic compounds and textile dyes.

Materials and Methods

Plant material

Banana, brinjal, potato, cabbage, and apple were obtained from a commercial source in Ahmednagar (Maharashtra, India).

Reagents

Catechol, was obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals

used were of analytical grade. Textile dye Blue 2RNL was collected from Manpasand textile industry, Ichalkaranji, India.

Partial purification of banana peel PPO

Banana peel (20 g) was homogenized in 50 ml 50 mM of phosphate buffer (pH 7.0). The homogenate was filtered through two layers of cheese cloth and then the filtrate was centrifuged at 14,000 rpm for 15 min at 4 °C. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to obtain 70% saturation and then centrifuged at 14,000 rpm for 15 min at 4 °C. The precipitate was dissolved in 50 mM phosphate buffer (pH 7.0). An enzyme extract was extensively dialyzed against the same buffer at 4 °C overnight. The dialyzed sample was used as the PPO enzyme source in the following experiments. For all other plant materials 20 g plant material was used for PPO enzyme preparation. Plant material (20 g) was homogenized individually in 50 ml 50 mM of phosphate buffer (pH 7.0). The homogenate was filtered through two layers of cheese cloth and then the filtrate was centrifuged at 14,000 rpm for 15 min at 4 °C. The filtrate was used as crude PPO enzyme source for respective plant materials.

Measurement of PPO activity

PPO activity was determined by measuring an initial rate of quinone formation as indicated by an increase in absorbance at 420 nm [12]. A Hitachi U-2800 spectrophotometer was employed throughout investigation. The sample cuvette contained 2.95 ml of 10 mM catechol solution in 50 mM phosphate buffer (pH 7.0) and 0.05 ml of the enzyme solution. The blank contained only 3 ml of substrate solution.

Protein determination

Protein concentration was determined according to the Lowry et al. [13] method with bovine serum albumin as standard.

Optimum pH and temperature of PPO enzyme

Optimum pH of the partially purified PPO was examined using 10 mM catechol as a substrate in a pH range 1.0-10.0 (KCl-HCl buffer; pH 2.0, glycine-HCl buffer; pH 3.0, sodium-acetate buffer; pH 4.0-5.0, sodium-phosphate buffer; pH 5.0-7.0, tris -HCl buffer; pH 8.0-9.0, NaHCO_3 -NaOH buffer; pH 10.0). All the buffers were prepared at 50 mM concentration. An optimum temperature for PPO activity was determined in the temperature range of 15-65 °C, and the reaction mixture was incubated for 10 min before addition of the enzyme.

Degradation of phenolic compounds

Four phenolic compounds (gallic acid, pyrogallol, tannic acid and phenol) were selected for the present study. Each compound (1 g/L) was incubated with banana PPO (8 U/mg protein/min) in 50 mM phosphate buffer, pH 7.0 at 35 °C. The control sets including inactivated enzyme preparation were also run.

Total phenol estimation

Total phenol estimation of untreated and treated samples was done by the method of Bhakta and Ganjewala [14]. A 0.1ml of each of the test sample was taken. The volume was made up to 7 ml in each test tube using distilled water. Folin-Ciocalteu (0.5 ml) reagent was added to each of the tube and shaken for 3 minutes. Then 1 ml sodium carbonate (35%) was added and the tubes were allowed to stand for one hour. The deep blue coloration developed was read for absorbance at 630 nm. The concentration of phenolics in the test extract was calculated using standard tannic acid (1 mg/ml) curve and expressed as milligram of tannic acid equivalents (TAE) per milliliter of the sample. Phenol concentration of the untreated sample was taken as 100% and phenol removal was calculated from the phenol remaining in the treated sample after each treatment.

Decolorization studies

Textile dye Blue 2RNL (40 mg/ml) was incubated with PPO (8 U mg/protein/min) in 50 mM phosphate buffer, pH 7.0 at 35 °C. The disappearance of the color by PPO activity was monitored at $\lambda_{\max}$ of the dye solution. The percent decolorization was calculated by taking the maximum absorbance of untreated dye solution as control (100%) [15]. Decolorization was monitored by UV-Vis spectroscopic analysis (Hitachi U-2800).

Determination of COD

The chemical oxygen demand (COD) was performed according to the standard methods for examination of waters and wastewaters [16]. The COD was determined by the closed reflux dichromate method. COD digestion tubes having capacity of 15 ml (pre-washed with dilute H₂SO₄) were taken and added various reagents along with sample as per the following sequence. 0.50 ml dye/phenolic compound solution or 0.50 ml treated sample was taken. To this 2.5 ml standard potassium dichromate digestion reagent was added slowly and mixed. Then 3.5 ml

sulfuric acid reagent was added through sides of the tubes and allowed it to go to the bottom. The content was mixed thoroughly and cooled. Then the tubes were transferred to the pre-heated COD digester at 150 °C and digested for 2 hrs. The blank was also tested by substituting distilled water for sample and same procedure was carried out exactly as sample. After digestion the contents of the COD digestion tube were transferred in 100 ml beaker. To this distilled water was added to make the volume to 50 ml. Then 1-2 drops of Ferroin indicator was added and titrated against 0.05 M ferrous ammonium sulphate solution.

Phytotoxicity study

In this experiment, effect of different concentrations of gallic acid and dye Blue 2RNL on germination of five plants namely *Cicer arietinum*, *Vigna sinensis*, *Vigna mungo*, *Vigna radiata* and *Triticum aestivum* was evaluated. The seeds were germinated in sterile 10 cm Petri dishes, layered with germination paper. Seeds were sterilized according to Somasegaram and Hoben [17] before transferring to the surface of the paper in the Petri dishes (10 seeds per plate). Seeds were irrigated with solutions of gallic acid and textile dye Blue 2RNL for each Petri dish at various concentrations. Also the seeds were treated with similar concentration of degradation products and its effect on germination was observed. Seeds germinated in distilled water irrigated Petri dishes were used as a control. All dishes were kept at room temperature for the period of six days. Seeds were considered germinated when the radical and hypocotyl together appeared. The germination percentage was estimated.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons. Graphpad software was used for this analysis.

Results and Discussion

Determination of polyphenol oxidase (PPO) enzyme activity in various sources

Polyphenol oxidase has been isolated from many higher plants such as potato, artichoke, pear, grape, banana, apple, apricot, plum, peach, and green olive [18,19]. In the present study six different plant sources namely brinjal, potato, cabbage, apple, banana peel, and banana pulp were screened for PPO activity. Out of which brinjal showed maximum activity (Table 1).

TABLE 1 PRESENCE OF PPO ENZYME ACTIVITY IN VARIOUS SOURCES

Sr. No.	Source	Enzyme activity (U/mg protein/min)
1	Brinjal	36.83 ± 3.17**
2	Potato	34.03 ± 3.27**
3	Cabbage	32.50 ± 2.50
4	Apple	26.56 ± 0.31
5	Banana peel	17.72 ± 0.91***
6	Banana pulp	2.66 ± 0.66

Values are a mean of three experiments ± SEM. Values significantly different from control at **P was <0.01, and ***P <0.001 were only considered.

Though brinjal exhibits maximum PPO activity, it is not used as source of enzyme in further studies. Brinjal is used as vegetable in daily diet in India therefore its use as enzyme source can increase the cost of process. Also it is not ethical to use food materials for getting enzyme preparation to treat wastewaters. Banana peel is purely a waste agricultural product and readily available. Considering all these points, an extraction of PPO from banana peel is more advantageous than extraction from other sources. By realizing this, the present study was focused on banana peel PPO.

Extraction and partial purification of PPO from banana peel

Extraction of PPO was carried out in 50 mM phosphate buffer (pH 7.0). Precipitates formed with $(\text{NH}_4)_2\text{SO}_4$ between 0-80% were tested to find the proper saturation point. The precipitate obtained from 70% $(\text{NH}_4)_2\text{SO}_4$ saturation contained most of the activity and this saturation was used for all extraction processes. An enzyme was purified 2.15-fold with 6.7% yield, by 70% $(\text{NH}_4)_2\text{SO}_4$ fractionation (data not shown). Purification of 2 to 8-fold has been reported for PPOs from different sources using $(\text{NH}_4)_2\text{SO}_4$ fractionation [20]. A comparison of purification fold for PPO enzyme extracted from various sources has been showed in Table 2.

TABLE 2 PRESENCE OF PPO ENZYME ACTIVITY IN VARIOUS SOURCES

Source	Purification fold (%)	Reference
Artichoke (<i>Cynara scolymus</i> L.)	1	Dogan et al. [21]
Banana (<i>Musa sapientum</i> L.) Peel	2	Yang et al. [22]
Seeds of Field Bean (<i>Dolichos lablab</i>)	2.1	Urszula et al. [23]
Butter lettuce (<i>Lactuca sativa</i> var. capitata L.)	3.0	Paul and Gowda [24]
Latent Persimmon Fruit	4.5	Nua et al. [25]

Optimum Temperature and pH

The polyphenol oxidase from banana peel was found to have maximum activity at 35 °C (Fig. 1). This result is very similar to report on the polyphenol oxidase of banana [26]. The temperature optimum of 35 °C for PPO isolated from source other than banana was also reported [27].

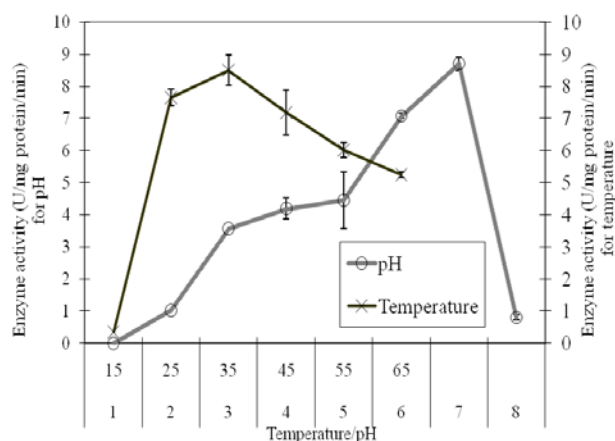


FIG. 1 EFFECT OF TEMPERATURE AND PH ON PPO ENZYME ACTIVITY

The pH activity profile for an oxidation of catechol by purified PPO is shown in Fig. 1. An optimum pH of 7.0 was found for the enzyme. Various researchers showed that 6.5-7.0 are an optimum pH values for banana PPO [26]. Differences in optimum pH with several substrates and sources have been reported for PPO [28].

Degradation of phenolic compounds by using partially purified PPO

Partially purified banana peel PPO degraded 4 various phenolic compounds (1 g/L each) in 24 h. The percentage degradation for each compound is shown in Table 3.

TABLE 3 PERCENT DEGRADATION OF DIFFERENT PHENOLIC COMPOUNDS AND REMOVAL OF COD BY BANANA PEEL PPO WITHIN 24 H

Phenolic compound (1 g/L)	Percent Degradation (%)	COD removal (%)
Gallic acid	100	97.5 ± 0.38**
Pyrogallol	85 ± 0.50	67.95 ± 0.13***
Tannic acid	80 ± 1.00**	63.94 ± 0.10
Phenol	70 ± 0.25	53.23 ± 0.68***

Values are a mean of three experiments ± SEM. Values significantly different from control at **P was <0.01, and ***P <0.001 were only considered.

Number of studies showed an application of fungal laccases in phenolic compound removal [29]. Precipitation of phenolic compounds as aggregates on reaction with phenol oxidases is a well-established phenomenon. Formation of these polymerization products was an indication of typical laccase activity [30]. In the present study no such aggregate formation was observed. This showed that the removal of phenolic compounds was due to breakdown of phenolic compounds by the action of PPO enzyme. The time course of gallic acid degradation by PPO enzyme was shown in Table 4.

TABLE 4 TIME COURSE OF DEGRADATION OF GALLIC ACID (1 G/L) BY BANANA PEEL PPO ENZYME

Time (h)	Percent Degradation (%)
4	15.67 ± 0.20***
8	38.30 ± 0.16**
12	65.00 ± 1.06***
16	84.90 ± 0.96***
20	97.00 ± 1.87***
24	100

Values are a mean of three experiments ± SEM. Values significantly different from control at **P was <0.01, and ***P <0.001 were only considered.

Enzyme was able to degrade major percentage of gallic acid (1 g/L) in first 12 h (65%) and 100% gallic acid was degraded in 24 h. In the control sets no degradation of phenolic compounds was observed. The remarkable removal of COD during degradation of various phenolic compounds was observed (Table 3). Due to an action of PPO enzyme on gallic acid, there was 97.5 (± 0.38**) % removal of COD in 24 h. In Gonzalez et al. [31] study, maximum effluent decolorization values and COD reduction attained after 7 days of fungal treatment were 73.3 and 61.7%, respectively. The present method costs less time for degradation therefore it is more advantageous than previously reported methods. Also PPO enzyme is more effective for removal of COD.

Effect of increasing concentration on degradation

Since 100% removal of gallic acid was achieved, the study was carried out to test an ability of PPO enzyme to degrade higher concentrations of gallic acid. There was a 68 (± 1.00**) % degradation of 5 g/L gallic acid in 24 hours (Table 5).

TABLE 5 DEGRADATION OF VARIOUS CONCENTRATIONS OF GALLIC ACID BY BANANA PEEL PPO ENZYME WITHIN 24 H

Gallic acid (g/L)	Percent Degradation (%)
1	100
2	98.5 ± 0.25**
3	95 ± 0.50**
4	85
5	68 ± 1.00**

Values are a mean of three experiments ± SEM. Values significantly different from control at **P was <0.01, and ***P <0.001 were only considered.

Also 100% degradation of this concentration of gallic acid was observed in 72 hours (data not shown). Kouroutzidou et al. [32] reported degradation of 1 g/L gallic acid in 23 days by using anaerobic sludge. The requirement of less time for gallic acid degradation and ability to sustain higher concentrations of gallic acid, explores the potential of PPO enzyme. According to Robles et al. [33], the total phenolic compound concentration in olive mill wastewater is 1.6 ± 0.18 g/L, but it may vary depending on the type and origin of the effluent. However, owing to the large volume and high pollution potential of the phenolic-rich effluent,

use of PPO enzyme for phenolic compound degradation is an attractive approach to industrial needs. There are number of reports in the literature on biodegradation of phenolic compounds [3,34]. An advantage of enzymatic approach over microbial degradation is that enzymes can react with a broad range of aromatic pollutants under dilute conditions. Our results indicate that by using PPO enzyme the rate of removal of pollutants is quite fast and is less sensitive to operational upsets as compared to the microbial flora. Duran and Esposito [10] also reported similar observations.

Decolorization and degradation of textile dye blue 2RNL

Polyphenol oxidase-mediated dye decolorization has been described previously [10, 18]. Time course of the decolorization was monitored for dye Blue 2RNL (40 mg/L). The partially purified PPO showed 89% decolorization of Blue 2RNL dye in 24 h (Fig. 2). The concentration of dye decolorized in the present study was comparable with other studies reported previously [35,36]. The spectrum of Blue 2RNL in visible region exhibits a main peak at 560 nm (Fig. 2). Absorption spectra for the treated dye (Blue 2RNL) exhibited decreased absorbance at various wavelengths in their spectra region as compared to untreated dye. The breaking down of the dye into smaller fragments can lead to a decrease in an absorbance of the visible spectra and in a colorless solution [37]. The decrease in absorbance spectra of treated dyes was also reported by several workers [15,18,36].

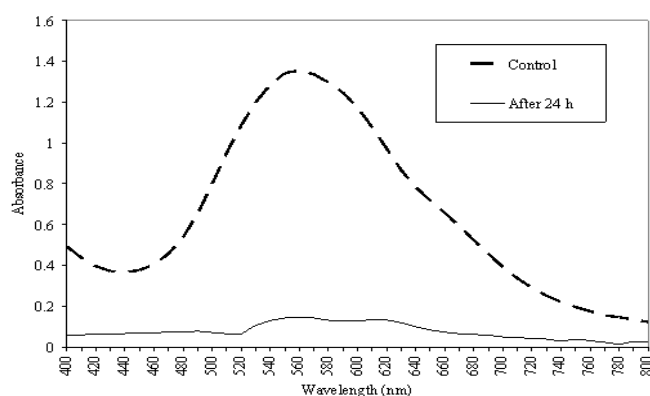


FIG. 2 DECOLORIZATION OF BLUE 2RNL BY PPO ENZYME

Removal of COD during decolorization of Blue 2RNL

The removal of COD during decolorization of dye Blue 2RNL was 92% (data not shown). The removal of COD suggested that degradation of dye resulted in the

formation of nontoxic compounds. Several earlier workers have described the loss of total organic carbon (TOC) during enzymatic decolorization of polluted wastewater [18]. Removal of COD during microbial decolorization of textile dyes was also reported previously [15].

Phytotoxicity study

The bioassay for phenolics and dye toxicity in this study was based on measuring the effect of these compounds on seed germination. Effect of gallic acid at various concentrations on five plant seeds was studied. The *Cicer arietinum* seeds showed only 20 ($\pm 0.15^{***}$)% germination at 5000 ppm concentration of gallic acid (Table 6).

TABLE 6 GERMINATION PERCENTAGE OF VARIOUS PLANT SEEDS

Plant	Germination percentage (%)		
	Control	Gallic acid (5000 ppm)	Product (5000 ppm)
<i>Cicer arietinum</i>	100	20 $\pm$ 0.15 ^{***}	100
<i>Triticum aestivum</i>	100	50 $\pm$ 0.06 ^{***}	100
<i>Vigna mungo</i>	100	50 $\pm$ 0.25 ^{***}	100
<i>Vigna sinensis</i>	100	60 $\pm$ 0.43 ^{**}	100
<i>Vigna radiata</i>	100	80 $\pm$ 0.17 ^{**}	100

Values are a mean of three experiments $\pm$ SEM. Values significantly different from control at ^{**}P was <0.01, and ^{***}P <0.001 were only considered.

The germination of these seeds was inhibited completely at 6000 ppm. The seeds of *Triticum aestivum*, *Vigna mungo*, *Vigna sinensis*, and *Vigna radiata* showed 50 ($\pm 0.06^{***}$), 50 ($\pm 0.25^{***}$), 60 ($\pm 0.43^{**}$) and 80 ($\pm 0.17^{**}$)% germination respectively at 5000 ppm. The germination of these seeds was inhibited completely at 7000, 7000, 8000 and 10000 ppm respectively (data not shown). The results of this study indicate that concentration of gallic acid higher than 5000 ppm was toxic for plants used. Similar study was conducted by Kaul [38] who demonstrated the toxicity of several phenolic compounds. At the same time the degradation product showed no such toxic effects on seed germination.

The relative sensitivity towards the dye Blue 2RNL and its degradation products in relation to germination of five plant seeds was studied. The *Cicer arietinum* seeds showed only 30% germination at 2000 ppm concentration of Blue 2RNL (data not shown). The germination of these seeds was inhibited completely at 2500 ppm. The seeds of *Triticum aestivum*, *Vigna mungo*, *Vigna sinensis*, and *Vigna radiata* showed 30, 50, 50 and 70% germination respectively at 2000 ppm. The germination of these seeds was inhibited completely at 3000, 4000, 4500 and 5000 ppm respectively (data not shown). In contrast to effect of dye, 100% germination was observed when seeds treated with degradation product (data not shown).

Conclusions

Enzymes can become prominent tools for solving an environmental contamination caused by phenolic compounds and textile dyes. In particular, wastewater treatment by plant polyphenol oxidases appears to be very promising. An ability of PPO enzyme to degrade gallic acid, pyrogallol, tannic acid, and phenol together with textile dye Blue 2RNL, confers to this enzyme a remarkable potential for its application in bioremediation and wastewater treatment, especially in detoxification of phenolic wastes. Use of banana peel as PPO source shows a practical applicability and usefulness for the treatment of industrial effluents in a cost effective manner.

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